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### DETECTION OF MUTATIONS IN HUMAN DNA

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## REVIEW

# Detection of Mutations in Human DNA

ULF LANDEGREN

*Efficient methods for the detection of mutations are of fundamental importance in research and in diagnostics. By detection of a DNA sequence alteration that cosegregates with a clinical phenotype in an affected family, the gene at fault may be identified and assigned a function. Mutation detection methods are also a rate-limiting factor for the clinical application of DNA diagnostics. Currently a large number of techniques are in use to scan for new mutations and to distinguish among previously established sequence variants. Here, some of the problems connected with mutation detection are discussed together with principles on which current and future mutation detection assays can be based.*

## Introduction

Mutations, or heritable alterations of the genetic material, are a substrate for evolution. They provide for biologic variation, acted upon by selective forces. In a clinical context, however, it is more natural to regard mutations as processes that threaten the integrity of the human organism. Accordingly, the task of the human geneticist is to identify deviations from normal nucleotide sequences and to assess the significance, if any, of this sequence variation.

Mutations occurring in somatic cells may become clinically manifest if they affect genes involved in growth control, resulting in tumor formation, or if they lead to dysfunction or death of critical cell populations in, for example, the brain. In the germline, by contrast, loss-of-function mutations in most genes can give rise to a detectable phenotype in humans [1]. The number of cell generations in the germline, from one gamete to a gamete in an offspring, may be around 20-fold greater in the male germline than in the female. On the other hand, in the female, an egg is formed after a second meiotic division that

may have lasted for 40 years. It is therefore not surprising that the incidence of different types of germline mutations and chromosomal aberrations depends on the parent of origin.

A majority of mutations, germline or somatic, are of little consequence to the organism since most of the genome appears to lack coding function. Even within protein-coding regions there is some tolerance to mutations due to the degeneracy of the genetic code and because the exchange of an amino acid may influence the function of a protein only slightly (see, for example, Strong et al. [2]) or not at all. With the development of increasingly efficient methods to scan large DNA segments for mutations, the need to predict the functional consequences of a mutation will become more pressing.

## Types of Mutations

While point mutations predominate among mutations in the human genome, individual genes may exhibit peculiar patterns of mutations and, accordingly, pose different diagnostic problems. In ~60% of cases of Duchenne muscular dystrophy, the mutation involves a deletion of a large segment of the gigantic dystrophin gene [3]. The recently elucidated mutation causing the fragile X syndrome is characterized by an increased copy number of a particular repeated sequence (CCG)<sub>n</sub> [4, 5]. Hereditary unstable DNA of this type may prove to be a more general phenomenon in human disease [6].

On a grander scale, chromosomal trisomy and unbalanced translocations also represent problems of copy number. So far, molecular genetic techniques have not have been used to any significant extent for the diagnosis of chromosomal aberrations in genetic and malignant disease [7], and cytogenetics remains the preferred technique to investigate this important genetic mechanism. In an individual with one mutated copy of a tumor suppressor gene, the remaining normal allele may be replaced by a second copy of the mutant allele in one cell per  $10^3$ – $10^4$ . Mechanisms causing this replacement include chromosomal nondisjunction, mitotic recombination, and gene conversion. In contrast, independent mutations, destroying the function of the remaining gene copy, are estimated to occur in one cell out of  $10^6$  [8].

Future DNA diagnostics may also be aimed at epigenetic effects. For instance, the methylation status of genes in different tissues may have a role in controlling expression of the corresponding proteins [9]. Somatic changes at the level of methylation of particular growth-controlling genes may prove to be

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an important factor in malignancy, requiring the development of new diagnostic strategies.

The detection of genetic changes by assaying the function of a protein offers the advantage that functional consequences of a mutation are readily apparent. Still, nucleic-acid-based techniques in most cases offer overwhelming advantages over other approaches to mutation detection.

## DNA Diagnostic Analyses

At the nucleic acid level, a standard set of detection techniques may be applied to mutations affecting any protein. Genomic sequences can be surveyed in any tissue samples, irrespective of when and where the protein is expressed. Genes may be analyzed as mRNA molecules in easily available tissues since tissue-specific genes tend to be illegitimately transcribed at a low level, in for example, peripheral blood cells [10, 11]. Also, when the gene causing a genetic disease remains unidentified, methods to monitor DNA sequence variants are useful to study the inheritance of disease through genetic linkage analysis.

Sensitive mutation detection techniques offer extraordinary possibilities for mutation screening. Analyses may be performed even before the implantation of a fertilized egg [12]. Prenatal diagnosis will be further simplified if techniques are established to isolate occasional fetal cells from maternal peripheral blood during pregnancy [13]. Increasingly efficient genetic tests may enable screening for oncogenic mutations in cells exfoliated from, for instance, the respiratory tract or the bladder in connection with health checkups [14].

## Methods for the Detection of Mutations

Mutations, involving as little as a single nucleotide, can be identified in a sample by physical, chemical, and/or enzymatic means. Most of the techniques may, and indeed often must, be preceded by target sequence amplification by, for example, the polymerase chain reaction (PCR). Methods for mutation detection are usefully divided into, on the one hand, scanning techniques suitable to identify previously unknown mutations in DNA segments of up to a few kilobases, and, on the other, techniques designed to rapidly detect, distinguish, or quantitate known sequence variants. A number of useful reviews describe the characteristics of current mutation detection methods [15-17]. Here some of the principles exploited to detect mutations are described as a back-

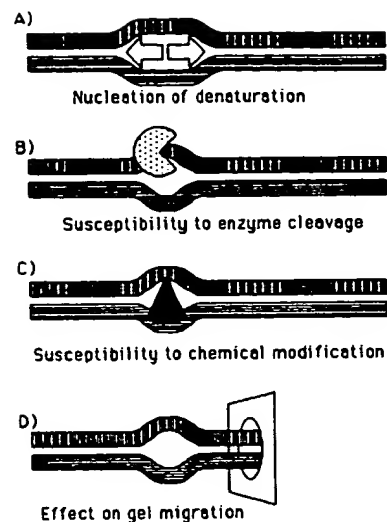


Figure 1. Heteroduplex methods to scan for new mutations in a DNA segment. The heteroduplex molecules are formed by hybridizing one strand from a normal sequence with the complementary strand from a mutant molecule. (A) A mismatched base pair will initiate partial or complete denaturation of two imperfectly complementary DNA strands when electrophoresed through an increasingly denaturing gel. (B) In positions where an RNA probe strand is not base-paired to its complement, RNase H can cleave the unpaired segment of the probe. (C) Chemical reagents, such as osmium tetroxide, hydrazine, or carbodiimide, may be used selectively to modify bases not engaged in base-pairing. (D) DNA duplexes mismatched in even just a single nucleotide position often exhibit altered migration through a hydrogel gel.

ground for a discussion of future DNA diagnostic techniques.

## Scanning Techniques for Mutation Detection

### *Influence of Mutations on Duplex Stability*

Mismatches in heteroduplexes of complementary DNA strands, derived from wild-type and mutant sequences, exhibit an abnormal denaturation behavior (Figure 1A). This phenomenon is exploited in the denaturing and temperature gradient gel electrophoresis (DGGE and TGGE, respectively) methods. Duplexes, mismatched in even a single nucleotide position, are likely to partially denature, and retard migration, at different positions when they are electrophoresed in an increasingly denaturing gradient gel [18-20]. The methods can detect any mutation in DNA segments of several hundred base pairs, and genetic variants in a complex pool of DNA samples may be quantitatively analyzed. No information is obtained regarding the precise location of a mutation,

but mutant forms are separated from the normal sequence and may thus be isolated and sequenced.

### *Susceptibility to Enzymatic Cleavage*

A heteroduplex of an RNA probe and a target strand may be cleaved by RNase A at a position where the two strands are not properly paired (Figure 1B) [21]. The site of cleavage can be determined by electrophoresis of the denatured probe. The technique has been successfully applied despite concerns that some mutations may escape detection. Recently, an interesting enzymatic activity involved in mismatch repair has been demonstrated in yeast, cleaving DNA strands at any mismatch [22].

### *Susceptibility to Chemical Modification*

Mismatched bases in a double helix are accessible to chemical modification (Figure 1C). Such modification can render the strands susceptible to cleavage at the site of the mismatch [23] or cause a polymerase to halt in a subsequent extension reaction [24]. The chemical cleavage technique has attained great popularity because it has the capacity to locate any mutation in target sequences of up to 2 kb and it provides information on the location of mismatched nucleotide(s). Using this technique, unknown mutations in the coagulation factor-VIII gene in hemophilic patients may be identified in 10 days [25].

### *Detection of Altered Nucleotide Composition*

In a less-explored strategy, the presence of a mutation in a DNA strand is visualized by substituting, during synthesis, one of the normal nucleotides with a modified version, altering the molecular weight of the product. A strand with an increased or decreased number of this modified nucleotide, relative to the wild-type sequence, will exhibit altered electrophoretic mobility, indicating the presence, but not location, of the mutation [26].

### *Effects on Gel Migration by Mutations*

Two very simple strategies visualize mutations in a DNA segment by revealing altered gel migration. In the single-strand conformation polymorphism technique (SSCP), mutations cause denatured strands to adopt different secondary structures, thereby influencing mobility during native gel electrophoresis [27]. Heteroduplex DNA molecules, containing internal

mismatches, can also be separated from correctly matched molecules by electrophoresis in so-called hydrolink gels (Figure 1D) [28].

### *DNA Sequence Analysis*

All of the above-mentioned techniques serve to locate a mutation in a limited segment of DNA and some of them enable a more precise localization within the segment. However, sequence analysis is required to unravel the effect of the mutation on the coding potential of the segment. Furthermore, to screen for the same mutation in other individuals of an affected family, nucleotide sequence information is required for most simple genetic tests. Similarly, DNA sequence data can be useful to monitor disease progression in the case of malignant disease or to detect residual malignant cells in the bone marrow before autologous transplantation [29]. As DNA-sequencing methods improve [30], the method of choice to scan for mutations in many cases probably will be sequence analysis.

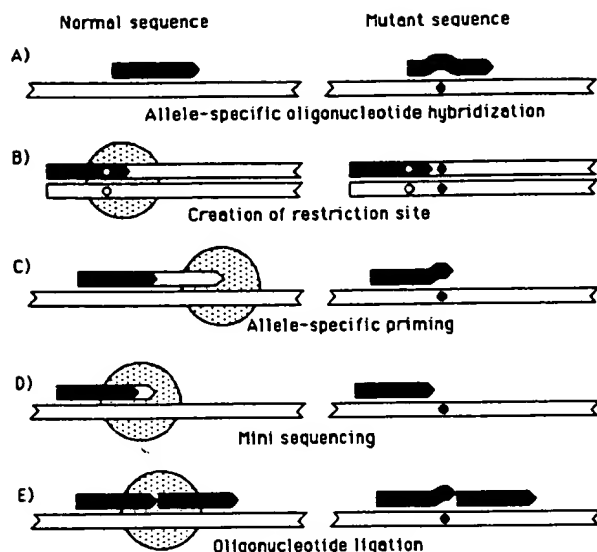
### *Methods to Reveal the Presence of Known Alleles*

A number of genetic diseases are caused by single, or a limited set of, mutations due to founder effects or advantages to heterozygous carriers. This pattern might become even more striking when we learn more about alleles related to common polygenic traits, including a large fraction of the important diseases in internal medicine [31, 32]. There will be an increasing clinical interest in monitoring sequence variants associated with, for example, altered metabolism of drugs or serving as genetic markers in forensic medicine. Also, in the diagnosis of infectious disease, identification of drug-resistant variant strains may require distinction between similar sequence variants.

A large number of techniques are available for the rapid and simple analysis of known sequence variants. Automation and economy are very important considerations for these types of analyses that may be applied, not only to individuals at risk, but for screening of the general population. Some of the principles exploited are discussed below.

### *Allele-Specific Oligonucleotide (ASO) Hybridization*

Mutations may be identified via their destabilizing effect on the hybridization of short oligonucleotide



**Figure 2.** Methods to distinguish between known sequence variants. The left panel represent a normal sequence to be distinguished from a mutant sequence in the right panel. A point mutation is indicated by a black dot. Synthetic oligonucleotide probes are illustrated in black. Enzymes are indicated as dotted circles. A sequence difference, introduced by using a partially mismatched PCR primer, is shown as a white dot. (A) The hybridization of an oligonucleotide probe to the target sequence is destabilized by a centrally located mismatch with the mutant target sequence. (B) Amplification of normal, but not mutant, target sequences with suitably designed PCR primers gives rise to amplification products that include a restriction site. (C) A mismatch in the 3' position of an oligonucleotide primer reduces its capacity to serve as an amplification primer. (D) An oligonucleotide primer stopping immediately short of a variable position can only be extended in the presence of the appropriate nucleotide, complementary to the variable position. (E) Two oligonucleotide primers, hybridizing in immediate juxtaposition, can be joined by a ligase provided there is no mismatch at the junction of the two oligonucleotides.

probes to a target sequence (Figure 2A) [33]. (For a discussion of the principles of oligonucleotide hybridization, see Wetmur [34].) After amplification of target sequences by using the PCR, short hybridization probes may be used that exhibit a prominent difference in their hybridization stability to matched and mismatched target sequences. A PCR product can be scanned for many possible sequence variants by investigating its hybridization to an array of immobilized oligonucleotide probes [35, 36]. A number of other strategies for nucleotide sequence distinction all depend on enzymes to identify sequence differences.

### Analysis of Restriction Sites

Restriction enzymes recognize segments of 4–8 nucleotides. Only around half of the nucleotide posi-

tions in a DNA segment can be monitored with a panel of 100 restriction enzymes [37]. As an alternative, artificial restriction enzyme recognition sequences may be created around a variable position by using partially mismatched PCR primers. In this manner, either the mutant or the wild-type sequence alone may be recognized and cleaved by a restriction enzyme after amplification (Figure 2B). The technique is useful for the detection of mutations in a minority population of tumor cells in a tissue sample [38, 39].

### Allele-Specific Priming

An oligonucleotide primer that is mismatched to a target sequence at the 3' penultimate position exhibits a reduced capacity to serve as a primer in PCR (Figure 2C). This property has been exploited by a number of groups as a means to distinguish DNA sequence variants during PCR. A problem with the method is that some 3' mismatches, notably G·T, are less inhibitory than others [40]. However, the allele distinction may be enhanced by designing the oligonucleotide so that it also includes a mismatch with both normal and wild-type sequences at the third positions from the 3' end. This results in two mismatched positions in the three 3' nucleotides of the primer hybridizing with one allelic variant, but only one inconsequential mismatch in the third position in from the 3' end when the primer hybridizes to the other allelic variant [41].

### Minisequencing

DNA polymerases may also be used to distinguish allelic sequence variants by investigating which nucleotide is added to an oligonucleotide primer, hybridizing immediately upstream of a variable position in the target strand (Figure 2D) [42, 43]. The assay enables excellent distinction between sequence variants; a mutation in as little as 0.3% of the cells in a sample may be detected [44].

### Oligonucleotide Ligation

Two oligonucleotide probes, hybridizing in immediate juxtaposition on a target strand, may be joined by a DNA ligase. Ligation is inhibited if there is a mismatch where the two oligonucleotide probes abut (Figure 2E) [37, 45–47]. The standardized nature of the oligonucleotide ligation assay (OLA) enables automated screening for known mutations in amplified DNA [48]. Since it depends on two independent

probes, the ligase-mediated detection reaction exhibits great specificity, enabling the detection of unique sequences in total human genomic DNA under standard reaction conditions [37]. Furthermore, when a thermostable ligase is used along with pairs of ligation probes that hybridize to both strands of a target sequence, allele-specific ligation products accumulate exponentially during temperature cycling (the ligase chain reaction, LCR) [47].

Using ligation probes, labeled with chelates of europium or terbium ions, two allelic sequences may be assessed simultaneously in an amplified DNA sample by sensitive time-resolved fluorometry [49]. The assay is further simplified by using a novel solid support [50] that enables the simultaneous handling of sets of 96 reactions with minimal pipetting steps (M. Samiotaki and U. Landegren, unpublished).

### Future Prospects

Several techniques for improved gene detection are just beginning to be explored [51]. With the development of novel amplification schemes, there are now good prospects for rapid and sensitive gene detection methods that are simple to perform and that operate at a fixed temperature [52, 53]. Provided the required specificity can be attained, such techniques may be suitable for doctor-office kits for diagnosis of, for instance, infectious disease. Probes establishing a triple-helical molecule with an undenatured target DNA segment by Hoogsteen base-pairing can be used to distinguish point mutations in a target sequence [54]. This type of third-strand probe or, alternatively, sequence-specific DNA-binding proteins, could possibly be applied in a nucleic acid biosensor, monitoring native DNA sequences in real time [55].

Future techniques for mutation detection may break down into two general categories. When little is known about what mutations to expect, improved methods for DNA sequence analysis may yield the information rapidly. Several groups are developing procedures to deduce nucleotide sequence by investigating what subset of all possible oligonucleotides of a given length can hybridize to a target segment under stringent conditions [56-58]. Sequencing by hybridization has great potential if applied to screening DNA samples for deviations from a known sequence. Simplified means of manufacturing arrays of densely immobilized sets of oligonucleotides—oligochips—are now becoming available [59]. Combined with technology for the production of microfabricated integrated sensors [60], efficient means to analyze the

hybridization patterns may result, leading to extremely rapid, simple, and relatively inexpensive techniques to scan genes for mutations.

The other category of mutation detection techniques addresses questions such as whether a particular target sequence is present in a sample, how many copies are present, or which among several known variants of this sequence can be demonstrated. In these cases, complete nucleotide sequence analysis will not be required. Critical features of such assays will be the possibility to analyze simultaneously a DNA sample with respect to many different sequences in an automated format [61]. It is desirable that a diagnostic technique exhibits sufficient specificity to be performed in total genomic DNA without prior target sequence amplification by PCR. A requirement for target amplification could place a low limit on the number of sequences that may be analyzed at one time in a DNA sample. Also, properties of the DNA sequence such as the methylation pattern may be obscured by amplification. Nonradioactive detection of even single target DNA molecules is an established technique in the microscopic format of chromosomal *in situ* hybridization [62, 63], and suitably miniaturized assays, perhaps using oligonucleotide chips, may well form the basis of tomorrow's techniques for monitoring known mutations and for scanning for new ones.

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